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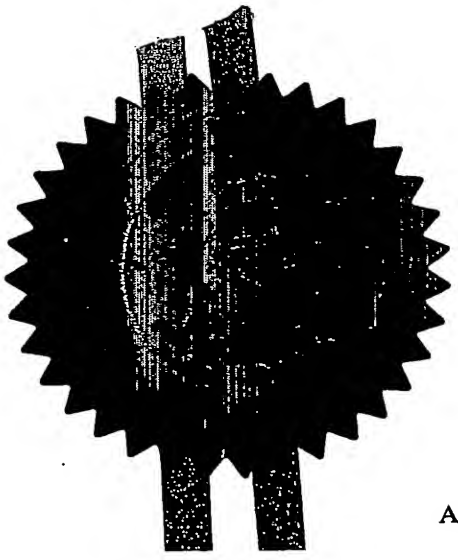

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Dated 7 April 2003

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1. Your reference — JPP128

2. Patent application number *(The Patent Office will fill in this part)* — **0205376.7** — - 7 MAR 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Royal Holloway University of London
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TW20 0EX

Patents ADP number *(if you know it)* 7547193001

If the applicant is a corporate body, give the country/state of its incorporation — ENGLAND

4. Title of the invention — Spore Germination

5. Name of your agent *(if you have one)* — Barker Brettell

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

138 Hagley Road
Edgbaston
Birmingham
B16 9PW

Patents ADP number *(if you know it)* 7442494002

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Country	Priority application number *(if you know it)*	Date of Filing *(day/month/year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature: Barker Brettell — Date: 06.03.02

12. Name and daytime telephone number of person to contact in the United Kingdom: James P. Peel — Tel: 0121 456 1364

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SPORE GERMINATION

This invention relates to the germination of spores and in particular, but not exclusively, to spores of Bacillus species of bacteria and uses thereof.

Infection is the leading cause of death in human populations. The two most important contributions to public health in the past 100 years have been sanitation and vaccination, which together have dramatically reduced deaths from infectious disease.

The development of improved vaccination strategies has always been of the utmost importance for a number of reasons.

Firstly, to provide better levels of immunity against pathogens which enter the body primarily through the mucosal surfaces. Vaccines are generally given parenterally. However, many diseases use the gastrointestinal (GI) tract as the primary portal of entry. Thus, cholera and typhoid are caused by ingestion of the pathogens *Salmonella typhi* and *Vibrio cholera* and subsequent colonisation at (*V. cholera*) or translocation (*S. typhi*) across the mucosal epithelium (lining the GI tract). Similarly, TB is initially caused by infection of the lungs by *Mycobacterium tuberculosis*. Immunisation via an injection generates a serum response (humoral immunity) which includes a predominant IgG response which is least effective in preventing infection. This is one reason why many vaccines are partially effective or give short protection times.

Secondly, to provide needle-less routes of administration. A major problem of current vaccination programmes is that they require at least one injection. For example tetanus vaccine. Although protection lasts for 10 years children are initially given three doses by injection and this

should be followed by a booster every 5 years. In developed countries many people will choose not to take boosters because of 'fear of injection'. In contrast, in developing countries where mortality from tetanus is high the problems lie with using needles that are re-used or are not sterile.

Thirdly, to offer improved safety and the minimisation of adverse side effects. Many vaccines consist of either live organisms which are either rendered non-pathogenic (attenuated) or are inactivated in some way. While in principle, this is considered safe there is evidence showing that safer methods must be developed. For example, in 1949 (the Kyoto incident) 68 children died from receiving a contaminated diphtheria vaccine (Health 1996). Likewise, in the Cutter incident of 1995 105 children developed polio. It was found that the polio vaccine had not been correctly inactivated with formalin. Many other vaccines, for example the MMR (measles-mumps-rubella) vaccine and the whooping cough vaccine (Health, 1996) are plagued with rumours of side effects.

Fourthly, to provide economic vaccines for developing countries where poor storage and transportation facilities prevent effective immunisation programmes. In developing countries where a vaccine must be imported it is assumed that the vaccine will be stored and distributed correctly. The associated costs of maintaining vaccines in proper hygienic conditions under refrigeration are significant for a developing country. For some vaccines such as the oral polio vaccine and BCG vaccine the vaccines will only survive for one year at 2-8°C (Health, 1996). The need for a robust vaccine that can be stored indefinitely at ambient temperature is a high priority now for developing countries. This type of vaccine should ideally be heat stable, able to withstand great variations in temperature as well as desiccation. Finally, a vaccine that is simple to

produce would offer enormous advantages to a developing country and would potentially be producable in that country.

It is an aim of the present invention to provide a spore in which said spore may be genetically modified to produce a medicament upon germination into a vegetative cell.

Accordingly, the present invention, provides a spore which is genetically modified with genetic code comprising at least one genetic construct encoding a antigen and a vegetative cell protein, as a chimeric gene.

It is an advantage of the present invention in that the use of spores to administer vaccines will eliminate the need for injections and the problems associated with needles in developing countries. In addition to this, spores are stable and are resistant to heat and desiccation, therefore overcoming problems of storing vaccines in developing countries. Spores are easy to produce, and can be done at low cost making the production of vaccines in accordance with the invention economical and finally, as a non-pathogen and its current use as an oral probiotic, the use of *Bacillus subtilis* makes this a safer vaccine system than those currently available.

It is a further advantage of the invention that the spores elicit an immune response at the mucosal membranes. This makes the vaccination more effective against mucosal pathogens e.g. *S.typhi, V.cholera* and *M.tuberculosis.*

A vaccine delivered at the mucosal surfaces will be more effective in combating those diseases which infect via the mucosal route. The mucosal routes of vaccine administration would include oral, intra-nasal and/or rectal routes.

It is a further advantage of the present invention in that when said spore is administered to an animal, said spore germinates into a vegetative cell, said vegetative cell expresses said chimeric gene, wherein said chimeric gene comprises said medicament and said protein in order to elicit an immune response against said antigen.

It is yet a further advantage of the present invention that mucosal immunity can be achieved using *B. subtilis* cells. It had been assumed that *B. subtilis* cells would have to be engineered to enhance their ability to interact with phagocytic cells (macrophages/dendritic cells) of the mucosa. This assumption is based upon the fact that some vaccine systems using heterologous antigen presentation use colonising bacteria (such as *Lactobacilli* or *Streptococci*) for antigen delivery. US 5 800 821 has specifically stated the need to express the *Yersinia pestis* invasion protein (Inv) in *B. subtilis* cells to promote interaction with the mucosa. Our present invention has shown this assumption to be unfounded and unnecessary.

Preferably the spore is of Bacillus or Clostridia.

The genetic modification is accomplished by transformation of a mother cell using a vector containing the chimeric gene, using standard methods known to persons skilled in the art and then inducing the mother cell to produce spores according to the invention.

The chimeric genes may be under the control of one or more of, each or independently, an inducible promoter, a promoter or a strong promoter or modified promoter. The chimeric genes may have one or more enhancer elements or upstream activator sequences and the like associated with them.

The vector may comprise an inducible expression system. The inducible expression system is such that when said spore germinates into a vegetative cell the antigen is not expressed unless exposed to an external stimulus e.g. pH or a pharmaceutical.

Generally the spore germinates in the intestinal tract. More preferably the spore germinates in the duodenum and/or the jejunum of the intestinal tract.

The genetic code may comprise DNA and/or cDNA. It will be appreciated that the term genetic code is intended to embrace the degeneracy of codon usage.

It has surprisingly been found not to be necessary to prime the spores to germinate prior to oral administration. This is particularly true of spores of the Bacillus species.

The spores are not heat inactivated prior to administration.

The vegetative cell only expresses a chimeric gene product after germinating from a spore. This may be achieved for example by, making a genetic construct of the antigen with a genetic construct of a protein expressed only in the vegetative state (e.g. the membrane associated protein OppA). This protein is not a spore coat protein.

The antigen is preferably at least a fragment of tetanus toxin fragment C or labile toxin B sub unit.

This aspect of the invention enables the antigen to be exposed to the human or animal body such that said antigen can elicit an immune response.

The antigen is preferably an antigen which, in use, is adapted to elicit an immune response.

The protein used may be any that are expressed only in the vegetative state. The protein may be a protein that is expressed in the cell barrier.

When we say a protein that is expressed in the cell barrier, we mean any protein (including lipoproteins and glycoproteins) that are expressed in, or in association with, the cell membrane, either intra-cellularly or extra-cellularly of the same; a protein expressed integrally with the cell membrane, a protein associated with the cell wall, either within the periplasmic space or externally of the cell wall or a protein expressed integrally of the cell wall.

This aspect enables a spore to be given orally to deliver the antigen. Alternatively, the spore may be administered via an intra-nasal or rectal route.

The antigen may be a chimera with different vegetative cell proteins. By having the genetic construct encoding the antigen with a genetic construct encoding one or more different vegetative cell proteins it may be possible to provide a temporal expression of the antigen. For example, the medicament may be expressed as a chimera with a vegetative cell protein that is expressed all the time, e.g. OppA, therefore providing a constant "dose" of antigen.

Alternatively, the genetic construct encoding the antigen may be with a genetic construct encoding a vegetative cell protein that is expressed intermittently and therefore upon expression of the chimera said chimera is capable of administering the medicament in a time-controlled manner.

The genetic construct encoding the medicament may also be with a genetic construct of a vegetative cell protein that is expressed initially at a high concentration but which then decreases over time, thus upon expression, the chimera is capable of administering an initial high dose of the antigen.

The temporal administration of doses could be customised by using, for example, one or more of the above genetic constructs.

Alternatively, the genetic construct encoding the antigen may be with a genetic construct encoding a soluble cytoplasmic vegetative cell protein.

When the antigen is expressed as a chimera with a soluble cytoplasmic protein, said soluble cytoplasmic protein may function to target the whole chimera to the periplasmic space for subsequent secretion by a passive mechanism, (e.g. diffusion). Alternatively, the soluble protein may target the chimera for secretion by an active mechanism, for example, by Type I, Type II or Type III secretion.

The genetic construct of the soluble cytoplasmic protein may wholly or partially comprise a signal sequence.

According to a second aspect, the present invention provides a spore which is genetically modified with genetic code comprising a genetic construct encoding an antigen and a signal sequence, wherein said signal sequence is adapted to target said antigen to a specific part of the vegetative cell. For example, the signal sequence may direct the medicament for secretion, for example active secretion (Type I, Type II or Type III secretion), or for post-translational processing by the vegetative cell, e.g. glycosylation.

The vegetative cells may lyse in the intestinal tract and subsequently release the antigen as a chimera.

When the antigen is expressed with a vegetative cell-barrier protein the antigen may generally elicit a localised immune response by the immune system in the immediate vicinity of the vegetative cell. Alternatively, when the antigen is expressed in the cytoplasm and the vegetative cells subsequently lyse and release the antigen or the antigen is secreted by the vegetative cells said antigen may generally elicit a diffuse immune response over a larger area than the immediate vicinity of the vegetative cell.

The spore, according to the present invention, may be genetically engineered to comprise one or more enzymes capable of transforming biological precursors, such that upon germination said one or more enzymes are expressed and synthesise one or more antigens by transformation of said biological precursors. For example by

a) processing a biological precursor, e.g. a hormone. The hormone may be a chimeric protein expressed in the vegetative cell e.g. a cell-barrier protein, which requires subsequent processing (i.e. release from the cell via an enzyme cleavage site) to be activated, or

b) the biosynthesis, or processing, of a non-protein compound, e.g. steroid hormones and painkillers synthesised from available biological precursor materials, or processing of a pro-drug into an active drug.

According to a further aspect, the present invention provides according to the invention in which said spore is genetically modified with genetic

code comprising at least one genetic construct encoding a medicament and a vegetative cell protein, as a chimeric gene.

The medicament may be one or more of: -

a) Proteins, including enzymes, antigens, antibodies, hormones or metabolic precursors;
b) Vaccines;
c) Endorphins and the like.

According to a further aspect, the present invention provides spores according to the invention for use as active pharmaceutical substances.

According to a further aspect, the present invention provides at least two different spores in which said at least two different spores express at least two different antigens or medicaments, according to the invention for use as active pharmaceutical compositions.

According to a further aspect, the present invention provides a spore according to the invention for use in the manufacture of a medicament for the treatment of a disease.

According to a third aspect, the present invention provides a composition comprising a spore according to the invention in association with a pharmaceutically acceptable excipient or carrier.

Suitable pharmaceutically acceptable carriers would be well known to a person of skill in the art.

According to a further aspect, the present invention provides a composition according to the invention for use in a method of medical treatment.

According to a further aspect, the present invention provides a spore according to the invention, for use in a method of medical treatment.

The invention also provides the use of the spore or of the composition according to the invention in the manufacture of the medicament for use in the treatment of a medical condition.

A method of medical treatment would comprise treating a medical condition e.g. a disease or administering a vaccine. Medical conditions for treatment by the invention include, for example, inflammation, pain, hormonal imbalances and/or intestinal disorders.

According to a further aspect, the present invention provides a method of medical treatment, which method comprises the steps of

a) Orally administering a spore according to the invention to a person or animal in need of medical treatment;

b) Said spore germinating into a vegetative cell in the intestinal tract;

c) Said vegetative cell expressing a medicament for use in the medical treatment.

The invention will now be described merely by way of example, with reference to the accompanying sequence listing and figures, of which:

Figure 1 shows a map of the pDG364 cloning vector showing the multiple cloning site, catgene and front and rear portions of the

amyE gene. Restriction sites that can be used for linearisation are indicated; nucleotide positions are noted in brackets.

Figure 2 illustrates the double-crossover recombinational event that generates a partial diploid using the cloning vector pDG364.

Figure 3a shows Western blotting of size fractionated (12% SDS-PAGE) proteins. A polyclonal antiserum to TTFC was used. Lane 1, non-recombinant strain PY79 vegetative cells, Lane 2, strain PY79 carrying *amyE::oppA-TTFC*. Lane 3, purified TTFC protein.

Figure 3b shows Western blotting of size fractionated (12% SDS-PAGE) proteins extracted from either the spore surface of non-recombinant PY79 spores (Lane 1), spores expresssing CotA::LTB (lane 2) and purified LTB protein. [note: The strain used for Lane 2 had the genotype *amyE::oppA-TTFC thrC::cotA-LTB]*

Figure 3c shows Western blotting using a polyclonal anti-TTFC serum to size fractionated proteins from sonicated extracts of vegetative cells. Lane 1, non-recombinant PY79 cells. Lane 2, *amyE::oppA-TTFC thrC::cotA-LTB* cells and Lane 3; purified TTFC protein.

Figure 4 shows anti-TTFC serum IgG titers following intraperitoneal immunisation with recombinant *B. subtilis* vegetative cells. Individual samples from groups of eight mice immunised intraperitoneally (↑) with $1x10^9$ wild-type (●) or OppA-TFFC expressing *B. subtilis* cells (Δ) were tested by ELISA for TTFC-specific IgG. Sera from a naïve control group (O) were

also assayed. The end-point titer was calculated as the dilution of serum producing the same optical density as a 1/40 dilution of a pooled preimmune serum.

Figure 5 shows anti-TTFC serum IgG titers following oral immunisation with recombinant *B. subtilis* spores. Individual samples from groups of eight mice immunised orally (↑) with $1.7x10^{10}$ wild-type (⊖) or OppA-TTFC recombinant *B. subtilis* spores (Δ) were tested by ELISA for TTFC-specific IgG. Sera from a naïve control group (O) were also assayed. The end-point titer was calculated as the dilution of serum producing the same optical density as a 1/40 dilution of a pooled preimmune serum.

Figure 6 shows anti-TTFC serum IgG titers following oral immunisation with recombinant *B. subtilis* spores. Individual samples from groups of eight mice immunised orally (↑) with $1.7x10^{10}$ wild-type (●) or OppA-TTFC CotA-LTB recombinant *B. subtilis* spores (Δ) were tested by ELISA for TTFC-specific IgG. Sera from a naïve control group (O) were also assayed. The end-point titer was calculated as the dilution of serum producing the same optical density as a 1/40 dilution of a pooled preimmune serum.

Figure 7 shows anti-LTB serum IgG titers following oral immunisation with recombinant *B. subtilis* spores. Individual samples from groups of eight mice immunised orally (↑) with $1.7x10^{10}$ wild-type (●) or OppA-TTFC CotA-LTB recombinant *B. subtilis* spores (Δ) were tested by ELISA for TTFC-specific IgG. Sera from a naïve control group (O) were also assayed. The end-point titer was calculated as the dilution of serum producing

the same optical density as a 1/40 dilution of a pooled preimmune serum.

Construction of recombinant genes

We have used the *oppA* gene for recombinant gene constuction. This gene is well studied and forms part of an operon. OppA acts as the receptor for the initial uptake of peptides by the oligopeptide permease (Opp). The OppA protein in *B. subtilis* is well expressed and is involved in competence as well as spore formation (it is referred to as SpoOK).

In the cytoplasmic membrane, fusion of a gene sequence to the 3'-end of *oppA* would allow expression of a recombinant protein (ProteinX) where OppA-Protein X is assembled into the membrane with the C-terminal domain (carrying the fused domain) exposed to the outer face of the membrane. In a Gram-positive this means that the antigen would be exposed to the space between the membrane and peptidoglycan wall.

Since *oppA* is involved in spore formation, any modification made to this protein must be made in *trans* to an intact copy. That is, one copy of *oppA* must be held intact on the chromosome. To achieve this, we use the *amyE* loci (encoding amylase) to carry chimeric genes. Thus, a recombinant *oppA-genX* chimera is placed at the *amyE* locus in cells carrying an intact *oppA* gene (and *opp* locus) at the normal chromosomal position. An alternative locus is *thrC* for which cloning vectors are available.

In the preferred embodiment of this invention, the Gram positive bacterium *Bacillus subtilis* is used. The excellent genetics associated with this organism and the intense study of its genome make it, after

Escherichia coli, the second most studied prokaryote. This organism is regarded as a non-pathogen and is classified as a novel food which is currently being used as a probiotic for both human and animal consumption. The single, distinguishing feature, of this microorganism is that it produces an endospore as part of its developmental life cycle when starved of nutrients. The mature spore, when released from its mother cell can survive in a metabolically dormant form for hundreds, if not thousands of years.

a) Construction of gene chimeras

i) ***amyE::oppA-TTFC.*** TTFC (tetanus toxin fragment C) is a 47 kDa component of tetanus toxin produced by *Clostridium tetani*. TTFC was fused to *oppA* and introduced at the *amyE* locus.

PCR was used to amplify i) appropriate sequences of the *tetC* gene (carried in vector pTet8) encoding the 47 kDa TTFC fragment, ii) the 5'-region of the *oppA* gene including its promoter. The *oppA* and *tetC* PCR products were fused using restriction digestion and ligation of 3' and 5' ends (using embedded cleavage sites in the PCR primers). The *oppA-TTFC* fragment was then cloned into the pDG364 vector (Figure 1) at the multiple cloning sites.

Figure 1 shows the plasmid pDG364 and this vector has been described elsewhere. The essential features of this vector are the right and left flanking arms of the *amyE* gene (referred to as *amyE* front and *amyE* back). Cloned DNA (ie, the *cot*-Antigen chimera) is introduced into the multiple cloning sites using general PCR techniques. The clone validated and the plasmid clone linearised by digestion with enzymes recognising the backbone sequences (eg, P*st*I). The linearised DNA is now used to transform competent cells of *B. subtilis* using selection for the antibiotic

resistance carried by the plasmid (chloramphenicol resistance). As shown in Figure 2, the linearised plasmid will only integrate via a double crossover recombination event using the front and back flanking arms of *amyE* for recombination. In the process the cloned DNA is introduced into the *amyE* gene and the *amyE* gene inactivated in the process. This procedure minimises damage to the chromosome and does not impair cell growth, metabolism nor spore formation.

The clone was verified by DNA sequencing across junctions and the vector linearised and then introduced into the chromosome of *B. subtilis* using a double crossover recombination (Figure 2). Selection for Chloramphenic-resistant and screening for amylase-negative colonies ensured a double crossover as shown in Figure 2 and is described elsewhere. Cells carrying this construct at *amyE* were tested for the presence of TTFC by Western blotting as shown in Figure 3 using a polyclonal antiserum to TTFC.

ii) ***amyE::oppA-TTFC thrC::cotA0-LTB.*** This construct carried two constructions placed at the *amyE* and *thrC* loci.

In this construction, we used a plasmid carrying a chimeric gene fusion of the *cotA* gene fused to the *Escherichia coli* 11 kDa Labile toxin Fragment B (LTB). PCR technology was used to amplify LTB and *cotA* sequences and fuse these together, in frame. CotA encodes a major protein 65 kDa from the spore coat surface layers. In the first step, the *cotA-LTB* chimera was constructed using the vector pDG1664. pDG1664 is similar to pDG364 (Figure 1) but carries the erythromycin-resistance gene (*erm).* Thus, selection for a double crossover recombination event is made by selection for Erm^R. The second important feature of pDG1664 is that insertion uses the front and back (left and right) arms of the *thrC* locus enabling insertion and disruption of the *thrC* locus. Using this strategy,

we made *thrC::cotA-LTB* cells, induced these to sporulate and then examined the spore coat proteins for the presence of CotA-LTB using a mouse polyclonal serum to LTB (Figure 3). Having demonstrated adequate expression of the CotA-LTB chimera on the spore surface we used chromosomal DNA of *thrC::cotA-LTB* to transform competent cells of a strain carrying *amyE::oppA-TTFC*. Selection was made for Erm^R and the transformants would carry two chimeric genes, *oppA-TTFC* and *cotA-LTB*. The presence of both chimeras was confirmed by Western blotting of vegetative cells with anti-TTFC serum and for spore coats proteins with ant-LTB serum.

b) Multiple antigen presentation

To achieve multiple antigen presentation on the spore coat, it is necessary to use either pDG364 and pDG1664 plasmid vectors. One chimeric gene is made in pDG364 and the chimera introduced at the *amyE* locus and a second chimera made in pDG1664 and introduced at the *thrC* locus. This can be achieved since each transformational event requires a separate drug-resistant selection.

We have used this approach to express LTB on the spore surface and TTFC within the vegetative cells. This feature is attractive and could be used for bivalent vaccinations. Alternatively we could use TTFC expression on the spore (fused to CotA) and also from the vegetative cells (fused to OppA) enabling even higher doses.

c) Strain Validation

In our approach, we do not reason that it is necessary to determine that the chimeric gene product is surface displayed, ie, on the surface-most layers of the cell. (This could be achieved using FACS analysis or some

other type of flow cytometry or using immunoflorscence). Our approach assumes that interaction of the vegetative cells with the mucosa must be achieved and in doing so, so long as an antigen is on or near the surface it will be able to stimulate immunity. This may well include cell-mediated immunity deriving from phagocytosis of the spore by macrophages or dendritic cells. In our rationale, it may actually be beneficial for the antigen to be partially protected within the cell envelope. Demonstration of immunogencity via mucosal immunisation is sufficient for further development.

d) Parenteral immunisation

Two immunisations were performed. First, intra-peritoneal immunisation of Black C57 inbred mice (groups of 8) with formalin-inactivated cells (approx. $5x10^9$) expressing OppA-TTFC. Figure 5 shows the serum IgG levels resulting from these immunisations and demonstrate the successful presentation and imnmunogencity of the OppA-TTFC chimera. To verify the immunogenicity of the double construct carrying OppA-TTFC and CotA-LTB we made both spores and vegetative cells and immunised (approx. $1x10^9$) by the IP route groups of 8 mice and followed the immune responses. Again high serum IgG levels were obtained by both routes.

e) Mucosal immunity

To achieve mucosal immunity, we used oral dosing of groups of 8 inbred Black C57 mice. We show some examples in Figures 5-7.

First, oral administration of high concentrations of spores ($1.7x10^{10}$) expressing OppA-TTFC (Figure 5). As shown, we were able to achieve serum anti-TTFC specific IgG responses at essentially protective levels

(usually reflected by titres higher than 10^3). The only way a IgG response could be achieved is if a significant level of spore germination had occurred leading to immunity.

Second, oral dosing of mice (Figures 6 and 7) with spores carrying CotA-LTB and OppA-TTFC showed high serum IgG levels against both LTB and TTFC. This showed that multiple antigens could be displayed and used to generate immunity and opens the way for development as bivalent vaccine.

Other Applications

1) This strategy could be used to display any biologically active molecule. For example, an enzyme for an industrial application.

2) In accordance with the invention, spores could also be used with adjuvants to enhance the immune responses of the germinated cells. These might include, cholera toxin, chitosan or aprotonin.

Any combination of spore coat protein for spore expression together with any cell envelope protein for expression in the vegetative cell. That is, we are not restricted to CotA or OppA. Primary candidates for spore coat expression that we have identified are CotA, CotB, CotC, CotD, CotE and CotG.

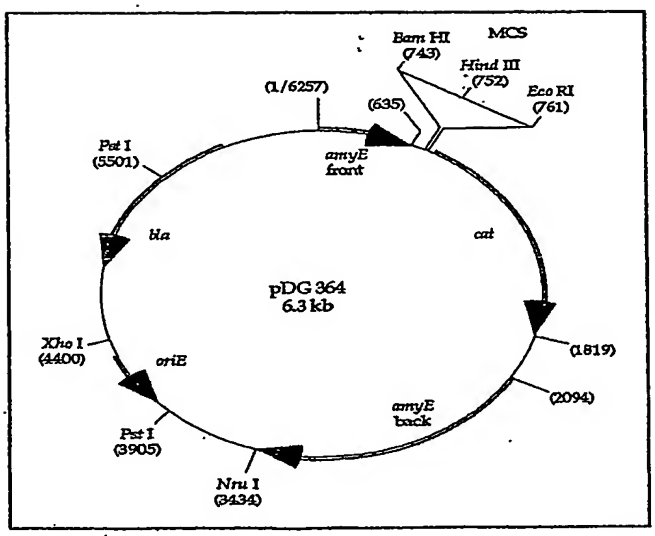
Other Cell Surface Presentation Routes

We have used the OppA proteins as an example for presentation based primarily on ease of use and high levels of expression. Other cell envelope proteins could also be used including proteins involved in chemotaxis, solute-uptake etc. The only criteria is:

i) that the antigen can be fused to an exposed domain of the protein,

ii) the protein is present in the membrane at high levels

To use these types of protein would require an empirical approach systematically attempting presentation one at a time. Another approach is to use proteins that are associated with the peptidoglycan of the cell envelope, ie, the wall itself. In many Gram positives there are a group of **"cell wall-anchored surface proteins"** that are covalently attached to both the cytoplasmic membrane and peptidoglycan of the cell wall.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(1/6257)
(635)
Pst I
(5501)
amyE
front
bla
cat
pDG 364
6.3 kb
Xho I
(4400)
oriE
(1819)
(2094)
amyE
back
Pst I
(3905)
Nru I
(3434)

FIGURE 1

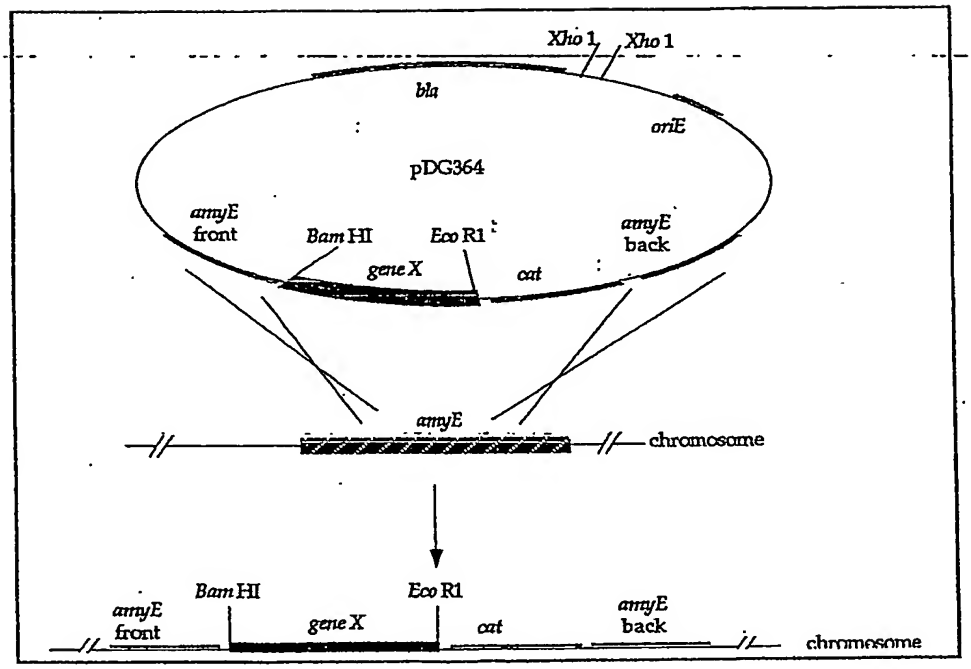
Xho 1
Xho 1
bla
oriE
pDG364
amyE
front
Bam HI
Eco R1
amyE
back
gene X
cat
amyE
chromosome
Bam HI
Eco R1
amyE
front
gene X
cat
amyE
back
chromosome

FIGURE 2

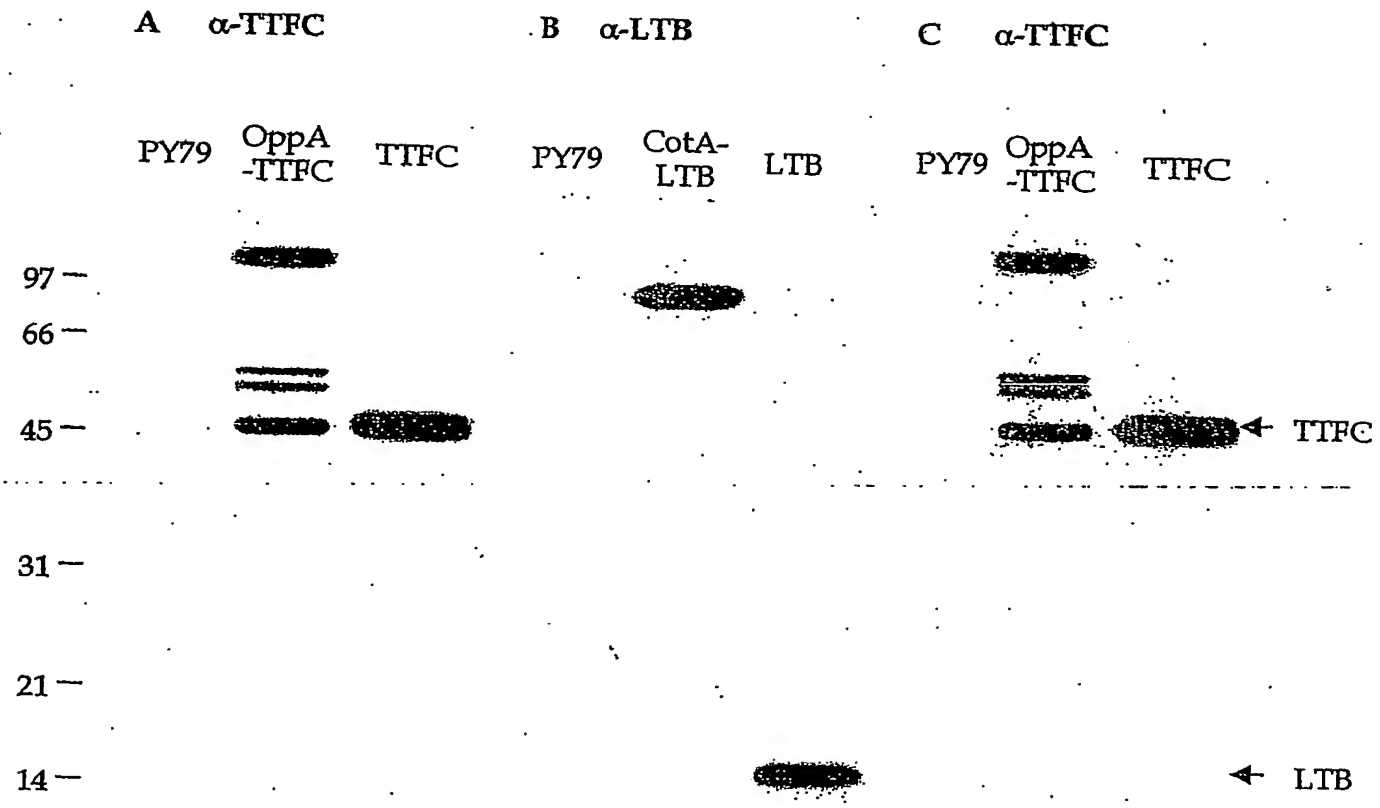
A α-TTFC
B α-LTB
C α-TTFC
PY79
OppA -TTFC
TTFC
PY79
CotA-LTB
LTB
PY79
OppA -TTFC
TTFC
97
66
45
31
21
14
TTFC
LTB

FIGURE 3

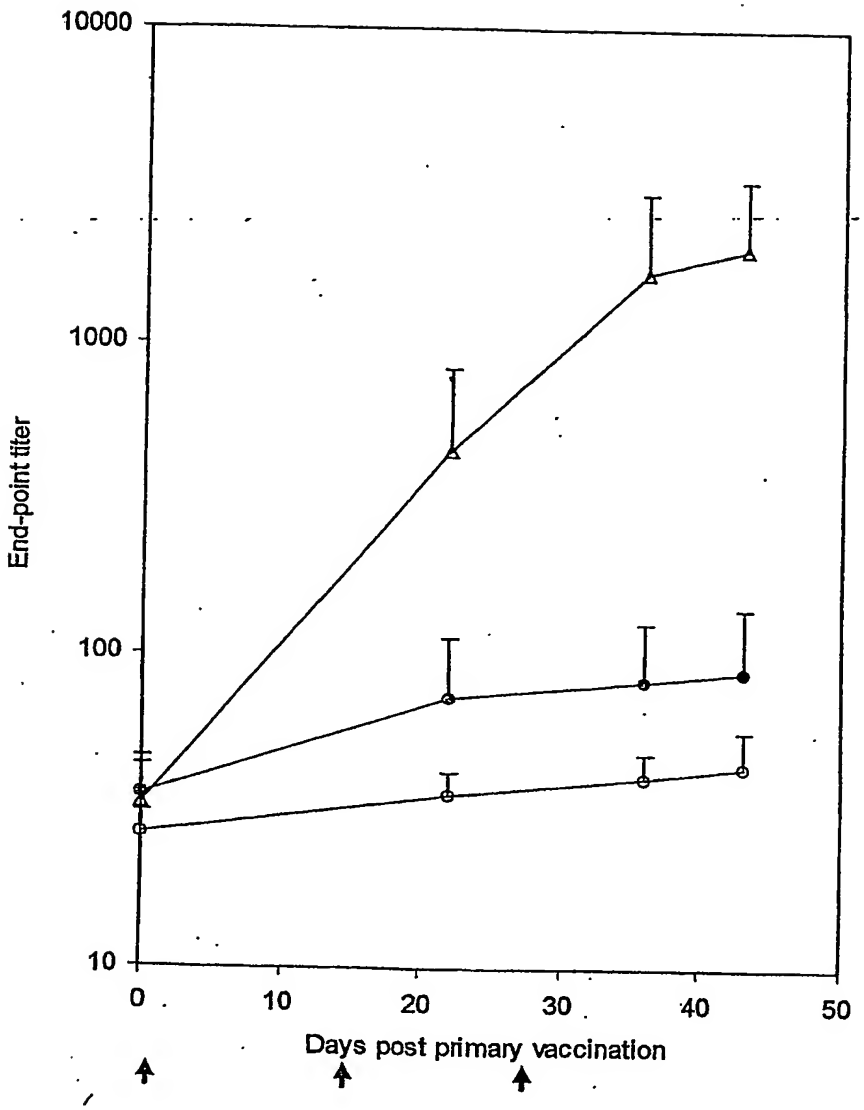
10000
1000
100
10
End-point titer
0
10
20
30
40
50
Days post primary vaccination

FIGURE 4

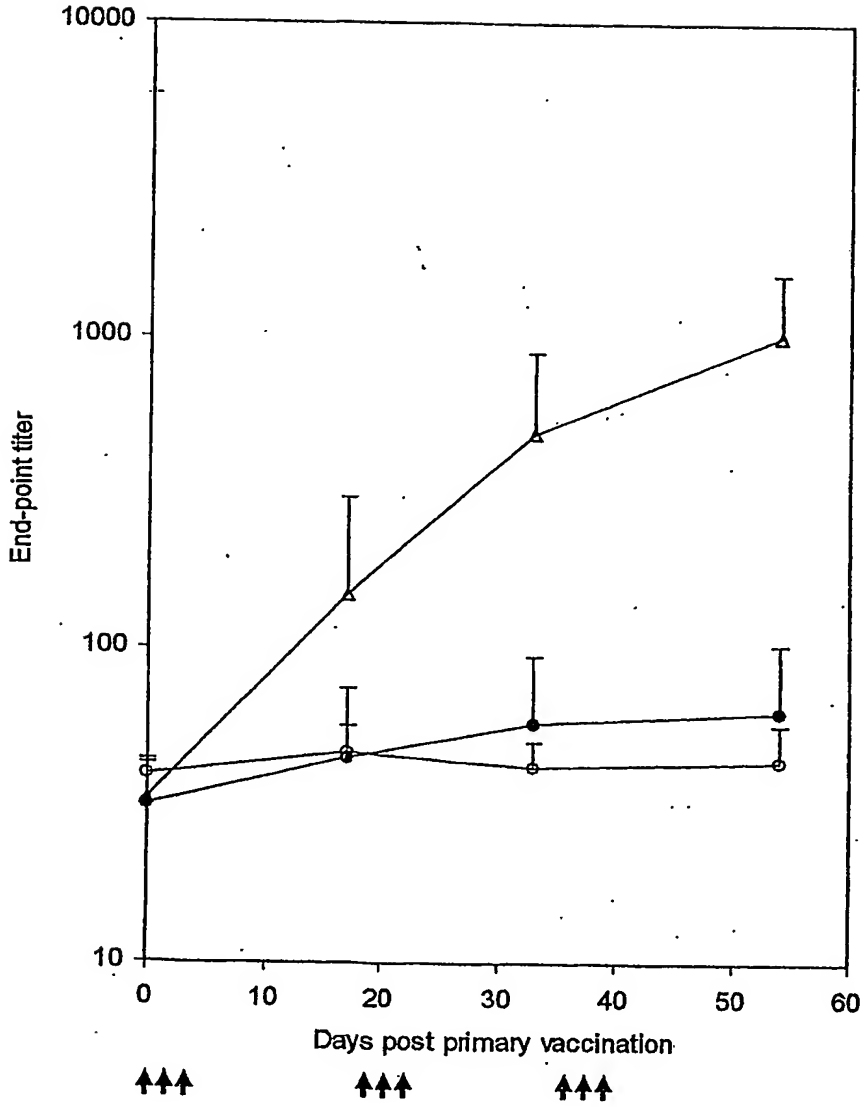
10000
1000
100
10
End-point titer
0
10
20
30
40
50
60
Days post primary vaccination

FIGURE 5

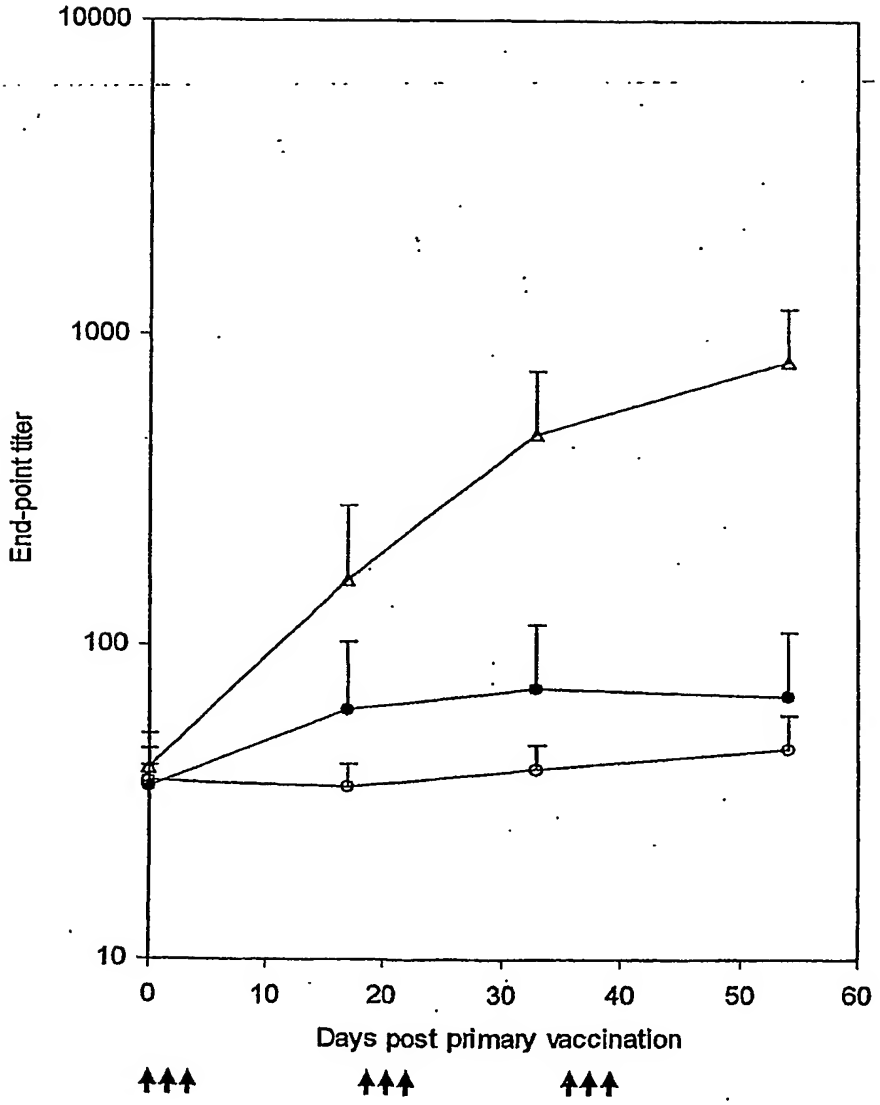
10000
1000
100
10
End-point titer
0
10
20
30
40
50
60
Days post primary vaccination

FIGURE 6

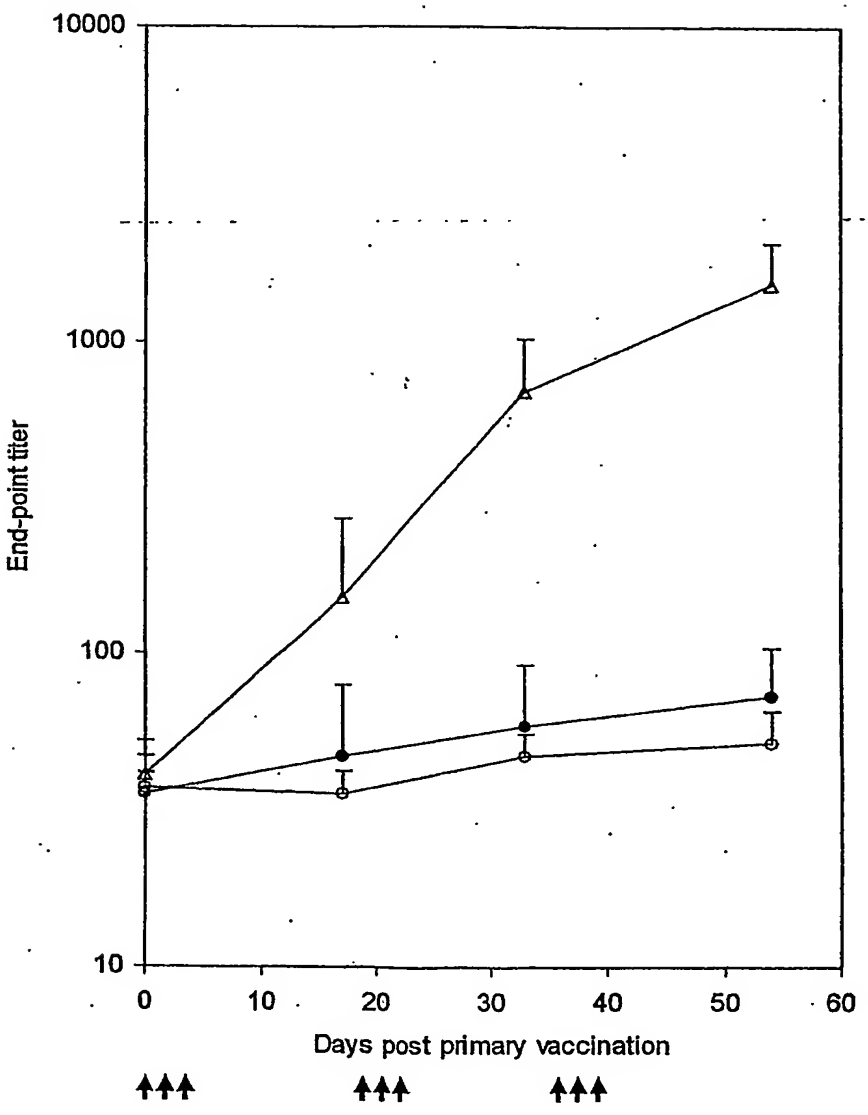
10000
1000
100
10
End-point titer
0
10
20
30
40
50
60
Days post primary vaccination

FIGURE 7